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Oxidative modification of rat eye lens proteins by peroxyl radicals in vitro: Protection by the chain-breaking antioxidants stobadine and Trolox

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Abstract

In an attempt to model the processes of free radical-mediated cataractogenesis, we investigated the oxidative modification of rat eye lens proteins by peroxyl radicals generated by thermal decomposition of 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) under aerobic conditions. When incubated with AAPH, the soluble eye lens proteins precipitated in a time-dependent manner. The insolubilisation was accompanied by the accumulation of protein free carbonyls and the diminution of sulfhydryls, yet the processes were shifted in time. The SDS-PAGE analysis of the AAPH-treated proteins revealed the presence of high molecular weight cross-links and, to a lesser extent, fragments. The aggregation and cross-linking of proteins along with the generation of free carbonyls was significantly inhibited by the chain-breaking antioxidants stobadine and Trolox. On the other hand, the AAPH-initiated sulfhydryl consumption was much less sensitive to the antioxidants studied. The results point to a complex mechanism of peroxyl-radical-mediated modification of eye lens proteins with implications for cataract development and they indicate a potentially protective role of antioxidants.

Keywords: 2,2′-Azobis(2-amidinopropane)hydrochloride; Cataract; Eye lens protein; Protein carbonyl; Peroxyl radical; Protein sulfhydryl

1. Introduction

Current evidence supports the view that cataractogenesis is a multifactorial process, in which a combination of several events induces an array of subtle post-translational modifications in the lens structural proteins, resulting eventually in lens opacification [1–5].

Human studies as well as in vitro and in vivo animal experiments strongly suggest that there is an association between increased oxidative stress and the development of cataract [6–10]. Oxidative free-radical damage is considered an initiating or very early event in the overall sequence leading to cataract [3]. Antioxidant supplementation was found to inhibit the development of diabetic cataract in rats [11–16], and several clinical studies pointed to a diminution of human senile cataract incidence after adequate supply of antioxidants in food [17–22]. The accumulation of protein carbonyls, a significant marker of protein oxidation, has been closely related to the development of both Emory mouse cataract [23] and human senile and diabetic cataracts [7,10,24,25]. Sulfhydryl oxidation is also thought to be one of the pathological events leading, through disulfide cross-linking and molecular aggregates, to protein precipitation and lens opacification [26–38]. In their study on human cataractous lenses, Boscia et al. [10] identified a threshold of protein oxidation above which clinically significant cataracts developed.

Isolated eye lens proteins (crystallins) treated with hydrogen peroxide, hydroxyl or peroxyl radicals, generated in the solution by different chemical or physical methods, are often used as experimental models of cataract [39–48]. A general concern is the relevance of such models to the authentic situation in vivo. Hydroxyl radicals have received the greatest attention owing to their established high reactivity. In contrast, the reactivity of peroxyl radicals is less understood in spite of the fact that their ability to

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oxidize cellular macromolecules is well documented [49–51]. Peroxyl radicals are produced in vivo in a variety of physiological and pathological processes [52–55] and may reach high steady-state concentrations in cells. Little information is available on the oxidation of eye lens proteins by peroxyl radicals, mechanisms that may have implications for cataract development.

The present study was undertaken to investigate oxidative modification of eye lens proteins in vitro by peroxyl radicals produced during the decomposition of the hydrophilic azo compound 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH) under aerobic conditions. We showed that exposure of the soluble fraction of eye lens proteins to AAPH led to an extensive loss of solubility, macromolecule cross-linking, formation of protein carbonyls and sulfhydryl loss. In addition, we recorded a protective role of the well-known chain-breaking antioxidants stobadine and Trolox in this model.

2. Materials and methods

2.1. Chemicals

2,4-Dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and electrophoresis grade chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA). 2,2′-Azobis(amidinopropane)dihydrochloride (AAPH) was from Fluka Chemie GmbH (Buchs, Switzerland). Prestained SDS-PAGE standard (21.2–108 kDa) was from Bio-Rad Laboratories (Hercules, CA, USA). Other chemicals were purchased from local commercial sources and were of analytical grade quality.

2.2. Eye lens proteins

Male Wistar rats, 8–9 weeks old, weighing 200–230 g, were used as eye lens donors for the preparation of soluble eye lens proteins. The investigation conforms with the Guide for the Care and Use of Laboratory Animals and was approved by the ethics committee and performed in accordance with Principles of Laboratory Animals Care (NIH publication 83-25, revised 1985) and Slovak law regulating animal experiments (Decree 289, part 139, July 9th 2003). The animals were killed by cervical dislocation (anesthetized with thiopental 65 mg/kg i.p.) and the eye globes were excised. The lenses were dissected and rinsed with ice-cold saline. The pooled lenses were homogenized in a glass homogenizer with a Teflon pestle in ice-cold phosphate buffer (20 mM, pH 7.4, 1.2 ml/each pair) saturated with nitrogen. The total homogenate was sonicated for 1 min and subsequently centrifuged for 30 min at 4 °C and 9000×*g*. The supernatant was dialyzed 20 h at 4 °C against 100-time-excess of 50 mM phosphate buffer, pH 7.4. The water-soluble eye lens proteins were analyzed for protein content according to [56] and stored deep frozen (below −20 °C) up to the time of processing (not exceeding 2 months).

2.3. Protein treatment with peroxyl radicals

Peroxyl radicals were generated by the thermal decomposition of AAPH at 50 °C [57,58]. The in vitro incubation mixtures of 2.5 ml total volume contained reagents added at the final concentrations in the sequence as follows: eye lens proteins (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), and AAPH (10 mM). The reaction mixture was incubated for different time intervals from 0 to 180 min at 50 °C.

To determine the loss of soluble proteins in the incubations, the reaction was terminated by cooling on ice. After centrifugation at 1500×*g* for 15 min at 4 °C, the supernatant was precipitated by TCA (1.25 ml, 30%). The pellets were washed with TCA (1 ml, 5%), and the precipitate was redissolved in a 1 ml solution consisting of Na_2CO_3 (10%) in NaOH (0.5 M). The final volume was made up to 2.5 ml by water and an aliquot of the solution was taken for protein determination [56].

For carbonyl determination and SDS-PAGE analysis, the reaction was terminated by cooling on ice and precipitated by ice-cold TCA (1.25 ml, 30%). The pellet obtained after centrifugation (1500×*g* for 15 min) was washed with TCA (1 ml, 5%).

For sulfhydryl determination, the incubation was terminated by cooling on ice and precipitated by ice-cold sulfosalicylic acid (SSA, 2.5 ml, 4%). The pellet obtained after centrifugation (1500×*g* for 15 min) was washed with SSA (1 ml, 2%).

2.4. Carbonyl determination

The content of free carbonyls in the lens proteins was determined by the procedure of Levine et al. [59] using the 2,4-dinitrophenylhydrazine (DNPH) reagent. Two TCA-precipitated, pelleted and washed aliquots of total proteins from incubations (approximately 3 mg of protein) were treated with 0.5 ml of 10 mM DNPH dissolved in 2 M HCl as a sample or with 0.5 ml of 2 M HCl as a control blank. The reaction mixtures were allowed to stand for 1 h at room temperature with stirring at 10-min intervals. Next, 0.5 ml of ice-cold 20% TCA was added and the samples were left on ice for 15 min. The precipitated proteins were subsequently washed three times with 1 ml of ethanol-ethyl acetate (1:1). The washed pellets were dissolved overnight in 1.8 ml of 6 M guanidine hydrochloride. Any insoluble material was removed by centrifugation at 1500×*g* for 15 min. The difference spectrum of the DNPH derivatives vs. HCl controls was scanned at 322–370 nm on a Hewlett Packard 8452 A Diode Array Spectrophotometer. Carbonyl content was calculated from the absorbance readings, using 22 000 l mol^{-1} cm^{-1} as the molar absorption coefficient at 368 nm. The final values were normalized to actual protein amount determined on

the basis of absorbance readings at 280 nm of parallel HCl-treated control blanks.

2.5. Sulfhydryl determination

Content of –SH groups in the lens proteins was determined using Ellman's procedure modified by Altomare et al. [8]. Two SSA-precipitated, pelleted and washed aliquots of total proteins from incubations (approximately 3 mg of protein) were dissolved in 0.2 ml of 6 M guanidine (pH 7.4) and read spectrophotometrically at 412 nm and 530 nm on Labsystems Multiscan RC Spectrophotometer, before and after a 30-min incubation in the dark with 50 μl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The content of protein sulfhydryls was calculated using a calibration curve prepared with reduced glutathione.

2.6. IC_{50} determination

The inhibition efficiencies of the antioxidants studied were characterized by IC_{50} values, i.e. the concentrations at which the antioxidants caused 50% inhibition of the parameter in question. The IC_{50} values were read from the linear portions of the semilogarithmic plots of I (%, percentage of inhibition) vs. antioxidant concentration. At least five concentration points of an antioxidant were chosen so that the corresponding I (%) values were minimally in the range from 20 to 80%. The final IC_{50} values are means±S.D. from three independent incubations.

2.7. Sodium dodecyl sulfate polyacryl amide gel electrophoresis

Pelleted protein samples (0.5 mg) were dissolved in a sample buffer solution of 5% SDS in a boiling water bath. Electrophoresis was conducted under non-reducing conditions according to Laemmli [60] with stacking (4%) and separating (10%) polyacrylamide gels. The gels were stained in 0.1% Coomassie blue and destained in 10% acetic acid in 25% methanol. The dried gels were scanned and the relative protein concentration of the bands was evaluated by UN-SCAN-IT® (Silk Scientific, Orem, Utah, USA) software.

3. Results

3.1. Kinetics of AAPH decomposition

Peroxyl radicals were generated by thermal decomposition of the hydrophilic azo compound 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH). Initial experiments were designed to monitor the kinetics of free radical generation. The kinetics of AAPH decomposition was determined by measuring the absorbance decrease at 366 nm, characteristic of the AAPH chromophore. As shown in Fig. 1, a linear decrease of the absorbance was observed in the time interval of 3 h. The slope of the degradation line at 50 °C gave the rate constant value k_d of 7.1×10^{-6} s^{-1}, which is in good agreement with that published by Niki [61] (1.36×10^{-6} s^{-1} at 37 °C), on assuming that a 5° increase in temperature doubles the rate of radical production.

The net flow v_i of peroxyl radicals reaching protein molecules in the solution and initiating protein damage can be calculated as follows:

$$v_i = 2e.k_d.c_{AAPH}$$

where e is the efficiency of peroxyl radical production and c_{AAPH} is the initial concentration of AAPH. Under similar conditions when liposomes were used as target macromolecules, the efficiency parameter e was determined as 0.48 [58]. Thus under the experimental conditions used, the absolute decrease of the azoinitiator observed during the first 3 h was less than 3%/h and a steady flow of peroxyl radicals, on average 245±7.35 nmol/ml/h, was attained during the whole incubation period.

3.2. Protein precipitation and oxidation

Fig. 2 (a–c) depicts the results obtained after the treatment of soluble eye lens proteins with AAPH for the time intervals indicated. The results represent the time course of protein precipitation (a), and time-dependent changes of carbonyl (b) and sulfhydryl content (c). The solutions remained clear and no changes in protein –SH and >CO groups were recorded in the incubations lacking the radical initiator AAPH.

As shown in Fig. 2a, AAPH-derived radicals led to a time-dependent formation of an insoluble product with the steepest increase between the minutes 15 and 60. The precipitate formed in this time interval was completely soluble in 6 M guanidine. The proportion of the precipitated protein attained approx. 75% after 120 min, when the curve reached plateau. The insoluble products formed beyond the minute 60 were only partially soluble in 6 M guanidine.

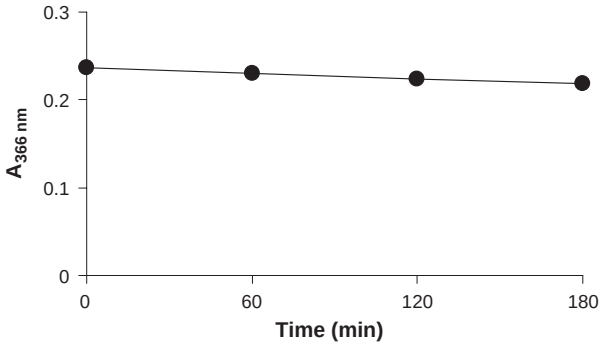

Fig. 1. Thermal degradation of AAPH as monitored by absorbance decrease at 366 nm. Solutions of 10 mM AAPH in 10 mM phosphate buffer (pH 7.4) were incubated at 50 °C. Results are mean values±S.D. from three incubations.

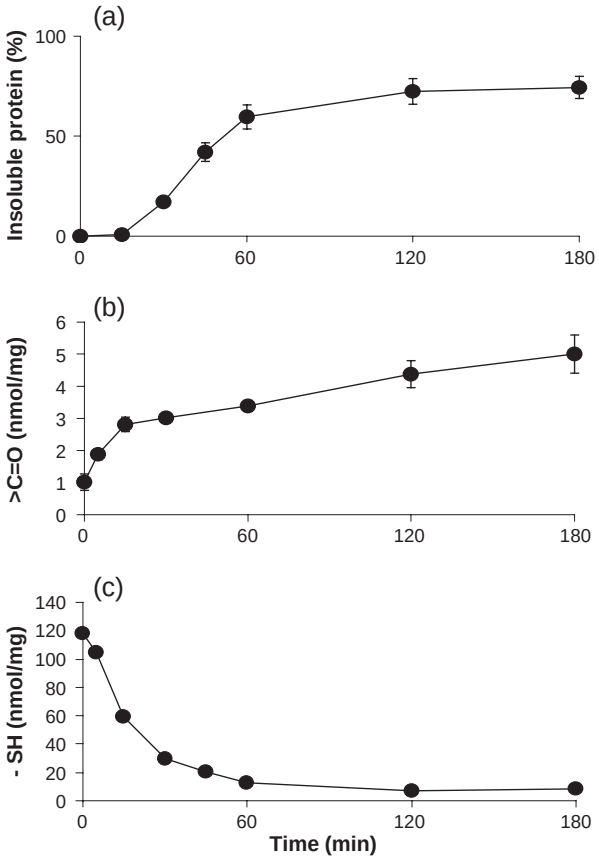

Fig. 2. Time-dependent changes of rat eye lens proteins induced by AAPH-derived peroxyl radicals in vitro. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated in the presence of 10 mM AAPH at 50 °C. Results are mean values±S.D. from three incubations.

Fig. 2b shows the increase in free carbonyl content of the eye lens proteins incubated in the presence of AAPH. In the time interval of 0–180 min, the level of the protein-bound carbonyls increased from 1.01±0.25 nmol/mg to 5.01±0.31 nmol/mg. The increase was very rapid during the first 15 min.

Finally, Fig. 2c shows a time-dependent decrease in the concentration of protein sulfhydryls simultaneously occurring with the free carbonyl accumulation. The level of the protein free –SH groups dropped from the initial 118±9 nmol/mg to 8.3±1.6 nmol/mg at minute 180. The decrease was very rapid during the first 15 min and reached a plateau after 60 min of incubation with the azo compound.

3.3. Changes in molecular weight (SDS-PAGE)

To determine if the AAPH-mediated precipitation and oxidation of the protein was associated with substantial molecular weight changes (either protein aggregation or fragmentation), the reaction mixtures were subjected to SDS-PAGE analysis. As shown in Fig. 3, the molecular weights of the native soluble eye lens proteins penetrating the stacking gel were in the range from 18 to 108 kDa (Lane 2). The high molecular weight (HMW) fraction (>108 kDa) was retained at the upper most portion of the resolving gel or failed to penetrate into the separating gel and was retained in the stacking gel.

In the oxidatively modified samples, the intensity of staining of protein bands corresponding to monomeric (18–32 kDa) and dimeric (42–47 kDa) crystallins was time dependently decreased compared to the respective bands of t_0 controls (Fig. 3). A concurrent increase of band intensities of HMW fractions was recorded in the stacking gel or in the start position of the separating gel; the densitometric evaluation of the gels showed an average increase in the overall HMW band intensity, expressed as percentage of total protein mass on the path, from 12.5±3.2% for t_0 samples to 36.1±6.9% for t_{180min} samples (Lane 7 vs. Lane 2). No changes in the proportions of the protein bands were observed in the incubations carried out in the absence of AAPH. These results show that peroxyl radical-mediated modification of soluble eye lens proteins resulted in the accumulation of higher molecular weight cross-links (most likely higher oligomers of eye lens crystallins). The observed limited solubility of the protein precipitate in saturated guanidine supported the notion of covalent cross-linking.

Protein fragments with molecular weights below 18 kDa appeared in the oxidatively modified samples, with the relative proportion not exceeding 8.0% in mean as quantified by densitometric evaluation of the gels.

3.4. Effect of antioxidants

In the incubations of soluble eye lens proteins with AAPH in the presence of stobadine or Trolox, efficient chain-breaking antioxidants, concentration-dependent inhibition of protein precipitation was observed (Fig. 4a). AAPH-induced changes of protein carbonyls were also markedly inhibited by the antioxidants studied (Fig. 4b). The

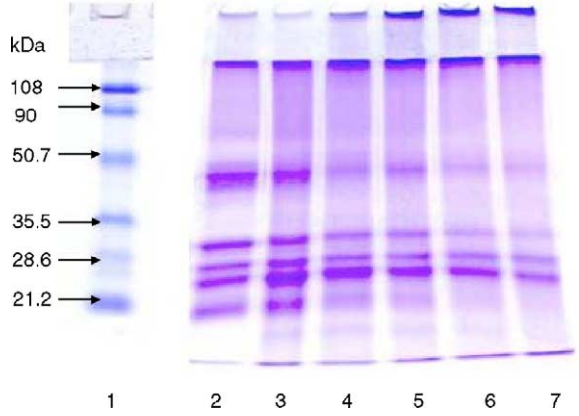

Fig. 3. Typical SDS-PAGE profiles of rat eye lens proteins oxidatively modified by AAPH-derived peroxyl radicals in vitro. Time dependence. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated in the presence of 10 mM AAPH at 50 °C. Lane 1—mass marker, Lane 2—t_0 control, Lane 3—time of incubation 30 min, Lane 4—45 min, Lane 5—60 min, Lane 6—120 min, Lane 7—180 min.

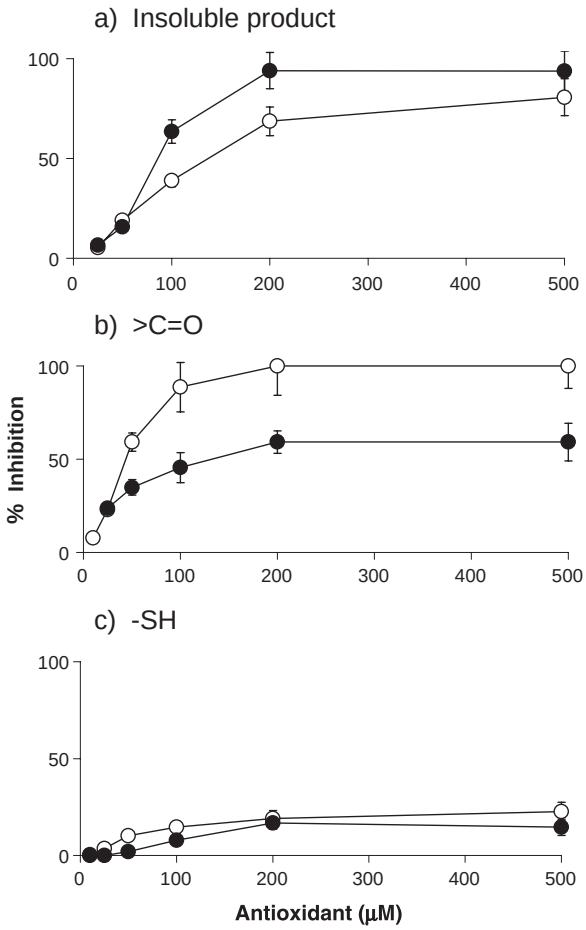

Fig. 4. Oxidative modifications of rat eye lens proteins induced by AAPH-derived peroxyl radicals in vitro. Effects of the antioxidants stobadine and Trolox. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated for 60 min (a) or 45 min (b), (c) in the presence of 10 mM AAPH and increasing concentrations of stobadine (○) and Trolox (●) at 50 °C. Results are mean values±S.D. from three incubations.

efficiencies of the inhibitions were characterized by determining the corresponding IC_{50} values as summarized in Table 1. As shown in Table 1, Trolox, in comparison with stobadine, was found to be a more efficient inhibitor of AAPH-induced precipitation of the soluble eye lens proteins. On the other hand, protein oxidation yielding free carbonyls was more efficiently inhibited by stobadine. As

Table 1
Soluble eye lens proteins of the rat exposed to AAPH-derived peroxyl radicals in vitro: effect of stobadine and Trolox

	IC_{50} (μM)	
	Stobadine	Trolox
Protein precipitation*	121±15	79±8
Protein oxidation yielding free carbonyl groups**	44±8	131±20
Sulfhydryl oxidation**	>500	>500

Soluble eye lens protein of the rat (0.8 mg/ml) were incubated with the azoinitiator AAPH (10 mM) and the antioxidants (stobadine 0–500 μM; Trolox 0–500 μM) in the phosphate buffer (10 mM, pH 7.4) at 50 °C. Time of incubation: *−60 min; **−45 min. Results are means±S.D. from three incubations.

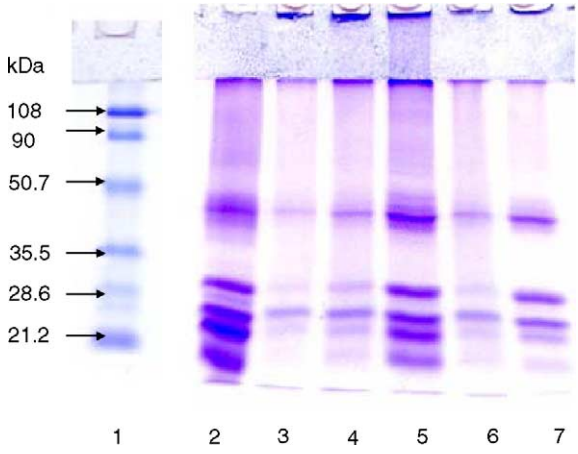

Fig. 5. Typical SDS-PAGE profiles of rat eye lens proteins oxidatively modified by AAPH-derived peroxyl radicals in vitro. Effects of the antioxidants stobadine and Trolox. Soluble eye lens proteins (0.8 mg/ml) in 10 mM phosphate buffer (pH 7.4) were incubated for 120 min in the presence of 10 mM AAPH at 50 °C. Lane 1—mass marker, Lane 2—t_0 control, Lane 3—no inhibitor, Lane 4—stobadine (0.1 mM), Lane 5—stobadine (1.0 mM), Lane 6—Trolox (0.1 mM), Lane 7—Trolox (1.0 mM).

shown in Fig. 4b, Trolox was less efficient than stobadine not only with respect to IC_{50} values but it also gave saturation of the inhibition effect at only 59.5±10.1%, while inhibition by stobadine reached 100% at the 500 μM concentration. Neither of the antioxidants studied gave 50% inhibition of sulfhydryl oxidation in the concentration range from 0 to 500 μM (Fig. 4c); at the highest concentrations studied (500 μM), the inhibitions were 22.7±4.8% for stobadine and 14.5±4.2% for Trolox.

As shown in Fig. 5, both stobadine and Trolox present in the incubations appeared to hinder peroxyl radical induced decrease of the intensity of monomer crystalline bands originally observed in the incubations without any antioxidant. Stobadine was apparently more efficient than Trolox.

4. Discussion

AAPH has been used extensively as a predictable and controllable source of free peroxyl radicals, physiologically relevant to biological systems [57,61–71]. In general, organic peroxyl radicals are less reactive than $^{\bullet}OH$, but with their longer half-lives they may be more damaging and more selective in their targets [51,53,64].

The present study was undertaken to determine the oxidative modification of soluble eye lens proteins by peroxyl radicals produced during the thermal decomposition of AAPH in aqueous solution. Within 180 min, about 75% of the total protein precipitated in the presence of the azoinitiator AAPH. Once precipitated, the proteins showed only limited solubility in concentrated guanidine, suggesting that, at least partially, true covalent cross-linking of the protein had occurred. SDS-PAGE analysis supported this assumption; however, the proportion of HMW products in

the AAPH-modified eye lens proteins reached only about 36% of total proteins in the incubations after 180 min. This discordance indicates that in addition to covalent cross-linking other mechanisms participated in the process of protein insolubilization induced by AAPH: Oxidation may induce conformational changes in protein structure leading to the disclosure (unfolding) of hydrophobic residues; e.g., Chao et al. [65] found that in vitro exposure of rat liver proteins to AAPH led to an increase in their surface hydrophobicity.

The kinetics of soluble eye lens protein insolubilization induced by AAPH-derived radicals was not in accord with the time-dependent changes of the markers of oxidative damage, free carbonyls and sulfhydryls. As shown in Fig. 2, the greatest changes of the biomarkers determined were observed in the time interval of 0–15 min, while the insoluble product formation started with a 15-min lag period. The processes also differ in their sensitivity to the antioxidants studied. Both stobadine and Trolox have been reported to be efficient scavengers of peroxyl radicals with similar efficacies, yet of different structure, molecular mechanism of action and affinity to lipid environment [58,72,73]. The anticataract action of stobadine was described in experimentally diabetic rats [74] while Trolox was found to prevent hyperglycaemia induced cataractogenesis in cultured rat eye lenses [75]. In comparing the inhibition curves shown in Fig. 4 and the IC_{50} values for the particular processes, as summarized in Table 1, there were apparent differences between the efficacy of inhibition of protein oxidation marked by free carbonyls and protein insolubilization, both for stobadine and Trolox. On the other hand, the AAPH-initiated sulfhydryl consumption was much less sensitive to the antioxidants studied. The results indicate that the peroxyl radical induced processes of eye lens protein insolubilization are dissociated from the processes of protein oxidation.

In spite of the fact that Trolox was found more efficient in inhibiting protein insolubilization than stobadine was, SDS-PAGE analysis showed that Trolox was apparently less efficient in preventing protein cross-linking. This discrepancy points to the participation of at least two alternative mechanisms involved in the precipitation of soluble eye lens proteins under the AAPH-derived radical attack as suggested above. Obviously, stobadine and Trolox differ in their inhibitory efficacies with respect to the particular mechanisms involved in protein precipitation. Stobadine seems to inhibit preferentially covalent cross-linking discernible by SDS-PAGE analysis, while the process of non-covalent aggregation appears to be more efficiently inhibited by Trolox.

In agreement with a general theory of protein peroxidation initiated by reactive oxygen species [49,64], we suppose that the antioxidants studied may affect the peroxyl radical-mediated modification of protein molecules both at the stage of initiation, by scavenging the primary peroxyl radicals originating from the thermal decomposition of AAPH in the presence of ambient oxygen, and at the stage of propagation, by scavenging the intermediate protein-derived radicals. A possible participation of a range of protein radicals in protein radical chain reactions has been demonstrated [76–78]. The affinity of an antioxidant molecule to the particular protein-derived radical species may vary broadly, depending on the nature of the amino acid residue involved and on its close neighborhood [53,64]. The chain process of protein oxidation becomes interrupted when a chain-breaking antioxidant effectively competes with the protein substrate for chain-carrying radicals. The water-soluble antioxidants stobadine and Trolox would be expected to have free access to these radicals.

Dean et al. [79] have suggested that along with peroxyl radicals, alkoxyl radicals may be generated in a smaller scale in the system of AAPH and protein. It is generally assumed that due to their high reactivity, alkoxyl radicals may be responsible preferentially for protein fragmentation [64,80–82] and thus would contribute to the complexity of the reactions described above. Indeed, under the experimental conditions used, we also observed a small proportion of the eye lens proteins to be fragmented. The result is in agreement with findings of Dean et al. [79], who recorded AAPH-induced fragmentation of bovine serum albumin in a similarly small scale.

In summary, the results shown indicate that AAPH-derived radicals precipitate, oxidize, cross-link, and fragment soluble eye lens proteins. In this respect, the AAPH-promoted modifications of eye lens proteins were found to be analogous to those reported for changes of crystallins in cataractous eye lenses, including aggregations and cross-linking leading to protein insolubilization [26,28,30,32,83], accumulation of reactive carbonyls [7,10,24,25], and decrease of free sufhydryls [10,26–38]. The peroxyl radical-mediated modification of eye lens proteins appears to involve a complex mechanism and should be further investigated along with implications for cataract development and a potential protective role of antioxidants.

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